

08/26/98

UTILITY PATENT APPLICATION TRANSMITTAL		Attorney Docket No.	N1205-003	Total Pages	21
For new nonprovisional applications under 37 CFR 1.53(b))		First Named Inventor or Application Identifier			
		HERBERT MARTIN WILSON <i>et al.</i>			
Express Mail Label No.					
APPLICATION ELEMENTS		ADDRESS TO: Assistant Commissioner of Patents Box Patent Application Washington, D.C. 20231			
MPEP chapter 600 concerning utility patent application contents.					
1. <input checked="" type="checkbox"/> Fee Transmittal Form (Submit an original, and a duplicate for fee processing)		6. <input type="checkbox"/> Microfiche Computer Program (Appendix)			
2. <input checked="" type="checkbox"/> Specification Total pages [21] (preferred arrangement set forth below)		7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)			
- Descriptive title of the invention		a. <input type="checkbox"/> Computer Readable Copy			
- Cross references to Related Applications		b. <input type="checkbox"/> Paper Copy (identical to computer copy)			
- Statement Regarding Fed sponsored R&D		c. <input type="checkbox"/> Statement verifying identity of above copies			
- Reference to Microfiche Appendix					
- Background of the Invention					
- Brief Summary of the Invention					
- Brief Description of the Drawings					
- Detailed Description					
- Claims					
- Abstract of the Disclosure					
3. <input type="checkbox"/> Drawing(s) (35 USC 113) (Total Sheets) [ ]		8. <input checked="" type="checkbox"/> Assignment Papers (cover sheet & document(s))			
4. <input checked="" type="checkbox"/> Oath or Declaration (Total Pages) [1]		9. <input type="checkbox"/> 37 CFR 3.73(b) Statement (when there is an assignee)			
a. <input checked="" type="checkbox"/> Newly executed (original or copy)		<input type="checkbox"/> Power of Attorney			
b. <input type="checkbox"/> Copy from a prior application (37 CFR 1.63(d))		10. <input type="checkbox"/> English Translation Document (if applicable)			
(for continuation/divisional with Box 17 completed)		11. <input type="checkbox"/> Information Disclosure Statement /PTO 1449			
[Note Box 5 below]		<input type="checkbox"/> Copies of IDS Citations			
i <input type="checkbox"/> DELETION OF INVENTOR(S)		12. <input type="checkbox"/> Preliminary Amendment			
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)		13. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) (Should be specifically itemized)			
5. <input type="checkbox"/> Incorporation by Reference (useable if Box 4b is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.		14. <input checked="" type="checkbox"/> Small Entity Statement(s) <input type="checkbox"/> Statement Filed in prior application, Status still proper and desired			
		15. <input type="checkbox"/> Certified Copy of Priority Document(s). (if foreign priority is claimed)			
		16. <input type="checkbox"/> Other:			
17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:					
[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No.:					
18. CORRESPONDENCE ADDRESS					
[ ] Customer Number or Bar Code Label or [X] Correspondence address below (Insert Customer No. or Attach bar code label here)					
Name	Robert J. Jondle, Reg. No. 33, 915				
Address	Rothwell, Figg, Ernst & Kurz Suite 701-East, 555 13th Street, N.W.				
City	Washington	State	D.C.	Zip Code	20004
Country	U.S.A.	Telephone	402-333-1550	Fax	402-333-1510

<b>FEE TRANSMITTAL</b>  <b>(Small Entity)</b>		Complete if Known	
		Application Number	09/
		Filing Date	
		First Named Inventor	Herbert Martin Wilson et al.
		Group Art Unit	
		Examiner Name	
Total Amount of Payment	\$457.00	Attorney Docket Number	N1205-003

**METHOD OF PAYMENT** (check one)

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any overpayment to Deposit Account Number 02-2135 in the name of Rothwell, Figg, Ernst & Kurz

☒ Charge any Additional Fee Required Under 37 CFR 1.16 and 1.17

☐ Charge for the Issue Fee Set in 37 CFR 1.18 at the Mailing of the Notice of Allowance

2. ☒ Payment Enclosed: Check

**FEE CALCULATION**
**1. FILING FEE**

Fee Description	Fee Code	Fee Paid
<input checked="" type="checkbox"/> Utility Filing Fee	201	395
<input type="checkbox"/> Design Filing Fee	206	165
<input type="checkbox"/> Plant Filing Fee	207	270
<input type="checkbox"/> Reissue Filing Fee	208	395
<input type="checkbox"/> Provisional Filing Fee	214	75
<b>SUBTOTAL</b>		<b>\$395</b>

**2. CLAIMS**

	Extra	Fee from below	Fee
Paid			
Total Claims	22 - 20 =	2 x \$11	= \$ 22
Independent Claims	2 - 3 =	x 41	= \$
Multiple Dependent Claims		+ 135	=
<b>SUBTOTAL</b>			<b>\$ 417</b>

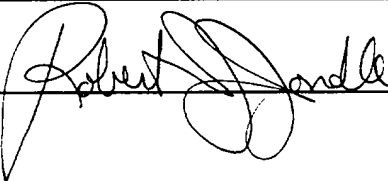
**FEE CALCULATION** (continued)

**3. ADDITIONAL FEES**

Fee Description	Fee Code	Fee Paid
<input type="checkbox"/> Surcharge - late filing fee or oath	205	65
<input type="checkbox"/> Surcharge - late provisional filing fee or cover sheet	227	25
<input type="checkbox"/> Non-English specification	139	130
<input type="checkbox"/> For filing a request for reexamination	147	2,520
<input type="checkbox"/> Requesting publication of SIR prior to Examiner action	112	920
<input type="checkbox"/> Requesting publication of SIR after Examiner action	113	1,840*
<input type="checkbox"/> Extension for reply within first month	215	55
<input type="checkbox"/> Extension for reply within second month	216	200
<input type="checkbox"/> Extension for reply within third month	217	475
<input type="checkbox"/> Extension for reply within fourth month	218	755
<input type="checkbox"/> Extension for reply within fifth month	228	1,030
<input type="checkbox"/> Notice of Appeal	219	155
<input type="checkbox"/> Filing a brief in support of an appeal	220	155
<input type="checkbox"/> Request for Oral Hearing	221	135
<input type="checkbox"/> Petition to institute a public use proceeding	138	1,510
<input type="checkbox"/> Petition to revive -unavoidable	240	55
<input type="checkbox"/> Petition to revive - unintentional	241	660
<input type="checkbox"/> Utility issue fee (or reissue)	242	660
<input type="checkbox"/> Design issue fee	243	225
<input type="checkbox"/> Plant issue fee	244	335
<input type="checkbox"/> Petitions to the Commissioner	122	130
<input type="checkbox"/> Petitions related to provisional applications	123	50
<input type="checkbox"/> Submission of Information Disclosure Statement	126	240
<input checked="" type="checkbox"/> Recording each patent assignment per property (times number of properties)	581	40
<input type="checkbox"/> Filing a submission after final rejection (37 CFR .129(a))	246	395
<input type="checkbox"/> For each additional invention to be examined (37 CFR 1.129(b))	249	395
<input type="checkbox"/> Other fee (specify)		

\* Reduced by Basic Filing Fee Paid

**SUBTOTAL \$ 40**

SUBMITTED BY		Complete (if applicable)			
NAME & REG. NUMBER	Robert J. Jondle, Reg. No. 33, 915				
SIGNATURE		DATE	August 24, 1998	DEPOSIT ACCOUNT USER ID	

## TITLE OF THE INVENTION

### TRANSGENIC PLANTS

## BACKGROUND OF THE INVENTION

5           The present invention relates to transgenic plants having improved traits and to a method for producing such plants. The method involves the incorporation of large fragments of DNA from one species of plant into a second species of plant and selecting plants with improved traits.

          The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by  
10       reference, and for convenience are referenced in the following text by author and date and are listed alphabetically by author in the appended list of references.

          The goal of plant breeding is to combine in a single variety/hybrid various desirable traits. For field crops, these traits may include resistance to diseases and insects, tolerance to heat and drought, reducing the time to crop maturity, greater yield, and better agronomic quality. With mechanical  
15       harvesting of many crops, uniformity of plant characteristics such as germination and stand establishment, growth rate, maturity, and fruit size is important.

          There are numerous steps in the development of any novel, desirable plant germplasm. Plant breeding begins with the analysis and definition of problems and weaknesses of the current germplasm, the establishment of program goals, and the definition of specific breeding objectives. The next step  
20       is selection of germplasm that possess the traits to meet the program goals. The goal is to combine in a single variety or hybrid an improved combination of desirable traits from the parental germplasm. These important traits may include higher yield, resistance to diseases and insects, better stalks and roots, tolerance to drought and heat, and better agronomic quality.

          Choice of breeding or selection methods depends on the mode of plant reproduction, the  
25       heritability of the trait(s) being improved, and the type of cultivar used commercially (e.g., F<sub>1</sub> hybrid cultivar, pure line cultivar, etc.). For highly heritable traits, a choice of superior individual plants evaluated at a single location will be effective, whereas for traits with low heritability, selection should be based on mean values obtained from replicated evaluations of families of related plants. Popular selection methods commonly include pedigree selection, modified pedigree selection, mass selection,  
30       and recurrent selection.

The complexity of inheritance influences choice of the breeding method. Backcross breeding is used to transfer one or a few favorable genes for a highly heritable trait into a desirable cultivar. This approach has been used extensively for breeding disease-resistant cultivars. Various recurrent selection techniques are used to improve quantitatively inherited traits controlled by numerous genes. The use of recurrent selection in self-pollinating crops depends on the ease of pollination, the frequency of successful hybrids from each pollination, and the number of hybrid offspring from each successful cross.

Each breeding program should include a periodic, objective evaluation of the efficiency of the breeding procedure. Evaluation criteria vary depending on the goal and objectives, but should include gain from selection per year based on comparisons to an appropriate standard, overall value of the advanced breeding lines, and number of successful cultivars produced per unit of input (e.g., per year, per dollar expended, etc.).

Promising advanced breeding lines are thoroughly tested and compared to appropriate standards in environments representative of the commercial target area(s) for three years at least. The best lines are candidates for new commercial cultivars; those still deficient in a few traits are used as parents to produce new populations for further selection.

These processes, which lead to the final step of marketing and distribution, usually take from eight to 12 years from the time the first cross is made. Therefore, development of new cultivars is a time-consuming process that requires precise forward planning, efficient use of resources, and a minimum of changes in direction.

A most difficult task is the identification of individuals that are genetically superior, because for most traits the true genotypic value is masked by other confounding plant traits or environmental factors. One method of identifying a superior plant is to observe its performance relative to other experimental plants and to a widely grown standard cultivar. If a single observation is inconclusive, replicated observations provide a better estimate of its genetic worth.

The goal of corn breeding is to develop new, unique and superior corn inbred lines and hybrids. The breeder initially selects and crosses two or more parental lines, followed by repeated selfing and selection, producing many new genetic combinations. The breeder can theoretically generate billions of different genetic combinations via crossing, selfing and mutations. The breeder has no direct control

at the cellular level. Therefore, two breeders will never develop the same line, or even very similar lines, having the same corn traits.

Each year, the plant breeder selects the germplasm to advance to the next generation. This germplasm is grown under unique and different geographical, climatic and soil conditions, and further  
5 selections are then made, during and at the end of the growing season. The inbred lines which are developed are unpredictable. Unpredictability results from the fact that the breeder's selection occurs in unique environments, with no control at the DNA level (using conventional breeding procedures), and with millions of different possible genetic combinations being generated. A breeder of ordinary skill in the art cannot predict the final resulting lines he develops, except possibly in a very gross and  
10 general fashion. The same breeder cannot produce the same line twice by using identical original parents and the same selection techniques. This unpredictability results in the expenditure of large amounts of research money to develop a superior new corn inbred line.

The development of commercial corn hybrids requires the development of homozygous inbred lines, the crossing of these lines, and the evaluation of the crosses. Pedigree breeding and recurrent  
15 selection breeding methods are used to develop inbred lines from breeding populations. Breeding programs combine desirable traits from two or more inbred lines or various broad-based sources into breeding pools from which inbred lines are developed by selfing and selection of desired phenotypes. The new inbreds are crossed with other inbred lines and the hybrids from these crosses are evaluated to determine which have commercial potential.

20 Pedigree breeding is used commonly for the improvement of self-pollinating crops or inbred lines of cross-pollinating crops. Two parents which possess favorable, complementary traits are crossed to produce an  $F_1$ . An  $F_2$  population is produced by selfing one or several  $F_1$ 's or by intercrossing two  $F_1$ 's (sib mating). Selection of the best individuals is usually begun in the  $F_2$  population; then, beginning  
25 in the  $F_3$ , the best individuals in the best families are selected. Replicated testing of families, or hybrid combinations involving individuals of these families, often follows in the  $F_4$  generation to improve the effectiveness of selection for traits with low heritability. At an advanced stage of inbreeding (i.e.,  $F_6$  and  $F_7$ ), the best lines or mixtures of phenotypically similar lines are tested for potential release as new cultivars.

Mass and recurrent selections can be used to improve populations of either self- or cross-pollinating crops. A genetically variable population of heterozygous individuals is either identified or created by intercrossing several different parents. The best plants are selected based on individual superiority, outstanding progeny, or excellent combining ability. The selected plants are intercrossed to produce a new population in which further cycles of selection are continued.

Backcross breeding has been used to transfer genes for a simply inherited, highly heritable trait into a desirable homozygous cultivar or inbred line which is the recurrent parent. The source of the trait to be transferred is called the donor parent. The resulting plant is expected to have the attributes of the recurrent parent (e.g., cultivar) and the desirable trait transferred from the donor parent. After the initial cross, individuals possessing the phenotype of the donor parent are selected and repeatedly crossed (backcrossed) to the recurrent parent. The resulting plant is expected to have the attributes of the recurrent parent (e.g. cultivar) and the desirable trait transferred from the donor parent.

Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several reference books (e.g. Allard, 1960; Simmonds, 1979; Sneep et al., 1979; Fehr, 1987).

Proper testing should detect any major faults and establish the level of superiority or improvement over current cultivars. In addition to showing superior performance, there must be a demand for a new cultivar that is compatible with industry standards or which creates a new market. The introduction of a new cultivar will incur additional costs to the seed producer, the grower, processor and consumer; for special advertising and marketing, altered seed and commercial production practices, and new product utilization. The testing preceding release of a new cultivar should take into consideration research and development costs as well as technical superiority of the final cultivar. For seed-propagated cultivars, it must be feasible to produce seed easily and economically.

Once the inbreds that give the best hybrid performance have been identified, the hybrid seed can be reproduced indefinitely as long as the homogeneity of the inbred parents is maintained. A single cross hybrid is produced when two inbred lines are crossed to produce the  $F_1$  progeny. A double-cross hybrid is produced from four inbred lines crossed in pairs ( $A \times B$  and  $C \times D$ ) and then the two  $F_1$  hybrids are crossed again ( $A \times B$ ) and ( $C \times D$ ). Much of the hybrid vigor exhibited by  $F_1$  hybrids is lost in the next generation ( $F_2$ ). Consequently, seed from hybrid varieties is not used for planting stock.

Recent advances in molecular biology have dramatically expanded the breeder's ability to manipulate the germplasm with which he is working. Genes controlling specific phenotypes, for example specific polypeptides that lend disease resistance or herbicide resistance, have been located within certain germplasm and isolated from it. Even more important has been the ability to take the genes which have been isolated from one organism and then introduce them into another organism. This transformation may be accomplished even where the recipient organism is from a different phylum, genus or species from that which donated the gene.

The development of these techniques has enabled the genetic engineering of desired traits by the introduction of exogenous genes into plant genomes. The uptake of new DNA by recipient cells has been accomplished by various means, including *Agrobacterium* infection (Nester et al., 1984), polyethylene glycol mediated DNA uptake (Lorz et al., 1985), electroporation of protoplasts (Fromm et al., 1986) and microprojectile bombardment (Klein et al., 1987). The transformed cells are selected, cultured and regenerated to form fertile transgenic plants. The transgenic plants are then introduced into the breeder's program to develop agronomically useful varieties which contain the new gene, using such breeding techniques as previously described. Transgenic plants of most, if not all, cultivated plant species have been produced using such genetic engineering techniques.

Tissue culture of corn is described in U.S. Patent Nos. 4,665,030, 4,806,483 and 4,843,005. Corn tissue culture procedures are also described in Green and Rhodes (1982). Transformation of corn is described in U.S. Patent Nos. 5,384,253, 5,489,520, 5,538,877 and 5,550,318. Corn transformation is also described by Prioli and Söndahl (1989) and Shillito et al. (1989).

Although transgenic plants are routinely prepared, the conventional process involves the isolation of a gene coding for a desired trait, the introduction of that gene into the particular germplasm of interest, regeneration of fertile plants and incorporation of the transgenic plants into the breeding program for analysis and development of agronomically acceptable varieties or hybrids. The identification and isolation of a gene coding for a desired trait can be very time consuming. In addition, the breeder must assess the effects of the inserted gene on the agronomic traits of the original germplasm, and must minimize such effects through breeding if plants with the new gene are to be agronomically acceptable to the grower.

It is desired to develop transgenic plants which does not require the isolation of specific genes for a desired trait. In accordance with the present invention, transgenic plants containing exogenous DNA having useful traits are prepared without the isolation of specific genes.

#### SUMMARY OF THE INVENTION

5           The present invention relates to transgenic plants having improved traits and to a method for producing such plants. The method involves the incorporation of large fragments of DNA from one species of plant into a second species of plant and selecting plants with improved traits. More specifically, DNA is isolated from one plant species, e.g. sorghum, and fragmented. The large fragments of DNA are then used to transform plant cells, e.g. corn, using conventional techniques, and  
10 transgenic plants are regenerated. The transgenic plants are grown in the field and analyzed for the expression of desired traits. Plants expressing desired traits are then introduced into a breeding program for the development of agronomically acceptable varieties and hybrids. Useful traits which can be introduced into recipient germplasm by this technique, include but are not limited to, increased resistance to drought conditions and heat stress, increased resistance to infestation by insect pests,  
15 increased standability, increased vigor, superior resistance to bacterial and fungal pests, superior combinability and superior yield.

#### DETAILED DESCRIPTION OF THE INVENTION

          The present invention relates to transgenic plants having improved traits and to a method for producing such plants. In general, the method involves the incorporation of large fragments of DNA  
20 from one species of plant, the donor species, into a second species of plant, the recipient species, and selecting transgenic plants with improved traits. More specifically, in accordance with the method of the present invention, DNA is isolated from the donor plant species, e.g. sorghum, and fragmented. The large fragments of DNA are then used to transform plant cells of the recipient plant species, e.g. corn, using conventional techniques, and transgenic plants are regenerated. The transgenic plants are grown  
25 in the field and analyzed for the expression of desired traits. Plants expressing desired traits are then introduced into the breeding program for the development of agronomically acceptable varieties and hybrids.



Useful traits to be identified in the field include: increased resistance to drought conditions and heat stress, increased resistance to infestation by insect pests including European corn borer and corn root worm, increased standability, increased vigor, and superior resistance to bacterial and fungal pests. Individual plants can also show superior combining ability and produce higher yields in combination with other inbred lines. All of the improvements will be attributable directly or indirectly to the presence of certain large fragments of DNA from the donor species integrated into the recipient species' genome, this attribution indicated by the segregation of the useful trait with the introduced donor fragment.

Introduction of uncharacterized large fragments of DNA isolated from one species and transformed into another can be used to improve any crop plant where the technology exists to produce such transformants. Examples include, but are not limited to, introgression of genes from: *Hordeum bulbosum* into *Hordeum vulgare* for the improvement of barley; *Avena barbata* into *Avena sativa* for the improvement of oats; *Aegilops ventricosa*, *A. umbellata*, *A. comosa*, *A. variabilis* or *A. sharonensis*, into *Triticum aestivum* for the improvement of wheat; *Nicotiana repanda* into *Nicotiana tabacum* for the improvement of tobacco; *Gossypium stocksii* into *G. hirsutum* for the improvement of cotton; *Lycopersicon hirsutum* into *Lycopersicon esculentum* for improvement of tomato; *Zea diploperennis*, *Z. mexicana*, *Tripsacum* spp, *Sorghum bicolor* into *Zea mays* for the improvement of corn; *Sorghum arundinaceum*, *Zea Mays*, *Trpisacum* spp into *Sorghum bicolor* for the improvement of sorghum. Examples are not limited to related species, nor to transfers between wild and cultivated species. Genes may be moved and tested in this manner from any plant species (donor) to any other plant species (recipient).

Large fragments (20 kb to 500 kb) may be introduced singly, or as part of the introduction of the entire genome. In accordance with the present invention, the production of transgenic plants is enhanced since no characterization at the DNA level is required. Transgenic plants having useful agronomic traits are selected in the field, i.e., the useful transgenic plants are determined by the phenotype of the transgenic plants in the field.

The present invention will be described with reference to sorghum as the donor species and corn as the recipient species. However, it is understood that this description is merely illustrative and the invention is applicable to any donor species and any recipient species. In accordance with the present

invention, transgenic corn containing fragments of sorghum DNA is produced. An average of 50% of the cultured embryos produced clones from which fertile transgenic plants are obtained. With this frequency of response, all 5000 fragments (with an average size of 150 kb) in a sorghum BIBAC library are introduced into the corn genome and each fragment is then evaluated for its effect on the phenotype of elite corn inbreds and hybrids. Co-ordinate gene expression on the fragments in the transgenic plants allows sorghum traits to be evaluated in corn. As previously described, precise knowledge of gene action and expression, and of the location of genes in the corn and sorghum genomes, is not required for the present invention to produce transgenic plants having improved agronomic characteristics. The requirement to evaluate specific sorghum sequences is met by the introduction of the entire sorghum genome in large fragments (averaging 150 kb in size) into the corn genome ensuring that all sorghum genes are tested. Several methods may be employed to analyze at the molecular level the integration of sorghum DNA into the corn genome. Methods include but are not limited to Southern blots, polymerase chain reaction (PCR) based assays, and/or DNA sequencing. Of these, Southern blot analysis is the most common method used to evaluate the presence of foreign DNA in a genome. PCR based methods require sequence information that may not be available, and sequencing of 150 kb is time consuming and expensive. A complication in the molecular analysis is that the sorghum DNA shares a high degree of homology with corn DNA. Consequently, it is likely that short probes (i.e., 50 to 200 nucleotides) or primers that hybridize only to sorghum DNA will be used in analyses. Briefly, Southern blots involve the isolation of total DNA from the transgenic plant, cutting the DNA with a restriction enzyme, separating the DNA on a agarose gel using electrophoresis, and transferring the DNA from the agarose gel onto a membrane that can then be probed with Sorghum DNA used to transform the plant. Regions such as 3' untranslated regions of genes tend to be unique and would work particularly well as probes for this type of analysis.

Transgenic regenerants are selfed or backcrossed and the progeny planted in rows in the field. Selectable markers such as the *bar* gene from *Streptomyces hygroscopicus* for resistance to the Liberty™ herbicide, the mutant maize *ahas* gene (serine to asparagine at amino acid position 621) for resistance to Pursuit™ herbicide, the CP4EPSPS gene from *Agrobacterium sp.* CP4 for resistance to Round-Up™ herbicide can be introduced with the sorghum fragments to facilitate selection in the field. Spraying with the appropriate herbicide can be used to identify the transgenic segregants. If selectable

markers are not introduced with the sorghum DNA, transgenic plants are grown in the field or greenhouse and analyzed for segregants in any measurable trait, including, but not limited to, maturity, drought resistance, cold hardiness, increased pest resistance, increased disease resistance, stalk strength, root strength, stay green, and the like. Transgenic segregants which show improved agronomic qualities can be selected for further evaluation both as inbreds and in hybrid combination. Generally, segregants with desirable changes are selected and selfed. Plants homozygous for the transgenes are then crossed with tester inbreds and hybrid yield evaluated. All transgenic segregants will potentially have an altered yield. In this manner, new inbreds and hybrids are produced having desirable agronomic characteristics.

## EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

### EXAMPLE 1

#### Construction of a Library in *Agrobacterium* Containing 20 kb Fragments of Sorghum DNA

Large molecular weight genomic DNA (100 kb) is isolated from Sorghum. Briefly, two grams fresh weight of tissue is frozen in liquid nitrogen and pulverized. Ground tissue is suspended in lysis buffer containing 20 mM EDTA, 10 mM Tris, pH 7.9, 1% Triton, 500 mM guanidine-HCL and 200 mM NaCl. This mixture is gently agitated at 37° - 46° C for 1-2 h. DNase-free RNase A is added to the concentration of 20 µg/ml and allowed to digest for 30 min at 37° C. Then proteinase K is added to the concentration of 0.8 mg/ml and all allowed to digest for 2 h. at 50° C. After centrifugation for 20 min. at 12 - 15,000 x g, total genomic DNA is isolated using Qiagen columns according to the manufactures recommendation. Fragmentation of the DNA is accomplished by partially digesting the DNA with the restriction enzyme HindIII as is common to the art. Partially digested DNA is fractionated on a 0.7% agarose gel and DNA fragments around 20 kb are isolated from the gel using the Qiaex II gel extraction procedure following the instructions by the manufacturer Qiagen. Partially digested plant DNA is then ligated to plasmid pSB12HBARGUS, which has been digested with HindIII and dephosphorylated to prevent self ligation. As a result the sorghum DNA is ligated between the *bar*

and *gus* expression cassettes, thus, expression of Bar and Gus protein is a good indicator of the inclusion of sorghum DNA into the corn genome. The resulting ligated products are transformed into transformation competent HB101 *E. coli* according to the manufacturer Gibco BRL's instructions. Individual colonies are analyzed for an insert by isolating plasmid DNA by standard mini-prep procedures, digesting it with HindIII restriction enzyme, and analyzing it on a 0.7% agarose gel. The plasmids are then mobilized into *Agrobacterium* strain LBA 4404 containing plasmid SB1, which harbors the *vir* genes C, G, and B, via triparental mating as described by Ditta et. al. (1980). During triparental mating pSB12HBARGUS containing sorghum DNA combines with SB1 via homologous recombination and is stably maintained thereafter in *Agrobacterium* and is ready to be used to transform corn.

## EXAMPLE 2

### Construction of a BIBAC Library in *Agrobacterium* Containing Greater than 100 kb Fragments of Sorghum DNA

The first step in constructing a library harboring sorghum-DNA-fragments larger than 100 kb requires the isolation of megabase size DNA as described by Woo et. al., 1995. Briefly, sorghum protoplasts are isolated from young leaf tissue, embedded in an agarose matrix using microbeads or agarose plugs, followed by cell lysis and protein degradation. DNA is made ready for cloning according to methods used to construct a bacterial artificial chromosome (BAC) library as described by Woo et. al., 1994. Briefly, the sorghum DNA is fragmented by partially digesting it with the restriction enzyme HindIII and separated on a low melting point agarose gel by pulse-field gel electrophoresis (PFGE). DNA ranging from 300-500 kb is isolated from the gel and used to ligate to the plasmid that has the essential features of a BIBAC vector as described by Hamilton, 1997, which includes a unique restriction site between the *bar* and *gus* expression cassettes (see Example 1). Plasmid is prepared for cloning by digesting with the same enzyme used to partially digest the megabase sorghum DNA and dephosphorylated as typically done to prevent self ligation. The ligation is performed according to Woo et al., 1994 and the resulting ligated products electroporated into electroporation competent *Agrobacterium* containing the plasmid pSB1, which harbors the *vir* genes C, G, and B. Randomly picked clones are analyzed for insert size by isolating the DNA by standard mini-prep procedures, digesting it with a restriction enzyme that liberates the sorghum DNA fragment,

and subjecting it to PFGE before it is used for the transformation of corn. Given an average insert size of 150 kb it would take 5000 clones to generate a genomic equivalent of the Sorghum genome given is  $7.5 \times 10^8$  base pairs.

### EXAMPLE 3

#### Introduction of Sorghum BIBAC into Corn Through *Agrobacterium*-Mediated Transformation

Sorghum BIBAC was introduced into corn through *Agrobacterium*-mediated transformation as modified from the procedure of Ishida et al. (1996). Briefly, immature embryos of the Stine inbred line 963 were aseptically removed from kernels of plants grown in a grow room (15 h photoperiod, 28° day and 25° night). Embryos were harvested 10 to 11 days after pollination when they were between 1 mm and 2 mm in length and then placed in 2 ml of LS-inf (Table 1) medium in an Eppendorf tube. The mixture was then stirred with a vortex mixer (Vortex Genie 2) at full speed for 5 seconds, the LS-inf medium removed and replaced with fresh medium, and then stirred again. All medium was then removed from the tube using a Pasteur pipette. Bacteria were collected with a platinum loop (enough to coat the wire of the loop) and thoroughly suspended in 1 ml of LS-infAS medium (Table 1) using a Pasteur pipette. The bacterial suspension was then introduced into the tube containing the embryos and the mixture stirred with a vortex mixer at full speed for 30 seconds. After this the embryos were allowed to stand for five minutes and were then transferred to the surface of LSAS medium solidified with agar (Table 1), care being taken to remove any accompanying liquid. Embryos were immediately oriented so that the scutellar surface was uppermost.

TABLE 1

LS Media Compositions

	<u>Ingredients/L</u>	<u>LSAS</u>	<u>LS-inf</u>	<u>LS-infAS</u>
5	MS salts/vits <sup>1</sup>	4.43 g	4.43 g	4.43 g
	Proline	700 mg		
	Casamino Acids		1.00 g	1.00 g
	Na <sub>2</sub> EDTA	37.3 mg	37.3 mg	37.3 mg
	2,4-D	1.5 mg	1.5 mg	1.5 mg
	MES	500 mg		
10	Thiamine HCl		1.0 mg	1.0 mg
	Sucrose	20 g	68.5 g	68.5 g
	Glucose	10 g	36.0 g	36.0 g
	Acetosyringone	100 µM		100 µM
	Phytagar	7 g		
15	pH	5.8	5.2	5.2

<sup>1</sup> MS salts – Sigma Plant Culture Catalogue ref. M5519

The embryos were then cultured in the dark at 19° C for 48 hours. After this time the plates were removed from the incubator and placed at 45° for 30 minutes. Then they were returned to the 19° C incubator for a further day. Following this the embryos were transferred to DN62ALC medium (Table 2) and incubated at 24° C for 5 days. Next, the embryos were transferred to DN62ALCB medium (Table 2) and incubated at 24° C for 14 days. For the next 14-day passage the cefotaxime concentration was raised from 50 mg/l (in DN62ALCB medium) to 250 mg/l. This medium - DN62ACB - allowed for a better control of the residual *Agrobacterium* cells contaminating the corn embryos. Embryos were then transferred back to DN62ALCB medium for a further 14 days. At this time, transformed corn clones could be recognized by their ability to grow as prolific Type II callus on the bialaphos-containing medium. Culture of the clones continued on DN62B medium (Table 2) for a further two weeks after which time the Type II callus was transferred to DNROB medium (Table 3) to initiate regeneration. After one to two weeks on DNROB medium, somatic embryos developed as individual structures. These embryos were allowed to mature for one to two weeks on a further passage on DNROB medium (Table 3) and were then transferred to 0.5M ABA6S medium (Table 3). Finally, they were transferred to MSOG medium or 1/2MS 0.1IBA medium (Table 3) where they germinated and formed plantlets. The plantlets were then transferred to tubes containing 1/2MS 0.1IBA medium where roots developed.

The plants were transferred to peat pots prior to going into the greenhouse. In the greenhouse, the plants were grown to maturity and seed collected either after backcrossing to Stine inbred 963 or after selfing.

TABLE 2

DN62 Media Compositions

<u>Ingredients/L</u>	<u>DN62B</u>	<u>DN62ALC</u>	<u>DN62ALCB</u>	<u>DN62ACB</u>
N6 salts <sup>1</sup>	3.98 g	3.98 g	3.98 g	3.98 g
N6 vitamins <sup>2</sup>	1 ml	1 ml	1 ml	1 ml
Asparagine	800 mg	800 mg	800 mg	800 mg
Myo-inositol	100 mg	100 mg	100 mg	100 mg
Proline	1400 mg	1400 mg	1400 mg	1400 mg
Casamino Acids	100 mg	100 mg	100 mg	100 mg
2,4-D	1 mg	1 mg	1 mg	1 mg
Sucrose	20 g	20 g	20 g	20 g
Glucose		10 g		
AgNO <sub>3</sub>		10 mg	10 mg	10 mg
Bialaphos	1 mg		1 mg	1 mg
Cefotaxime		50 mg	50 mg	250 mg
Gelrite	3 g	3 g	3 g	3 g
pH	5.8	5.8	5.8	5.8

<sup>1</sup> N6 salts – Sigma Plant Culture Catalogue ref. C 1416

<sup>2</sup> N6 vitamins: 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 1 mg/l thiamine HCl (Chu, 1978).

TABLE 3

Regeneration Media Compositions

<u>Ingredients/L</u>		<u>DNROB</u>	<u>0.5MABA6S</u>	<u>MSOG</u>	<u>1/2MS 0.1IBA</u>
5	MS Salts <sup>1</sup>	4.43 g	4.43 g	4.43 g	2.215 g
	Asparagine	800 mg			
	Proline	1400 mg			
	Na <sub>2</sub> EDTA	37.3 mg	37.3 mg	37.3 mg	37.3 mg
	Casamino Acids	100 mg			
10	Nicotinic Acid	0.5 mg			
	Gibberellic Acid			0.1 mg	
	Chloramben		0.5 mg		
	Absciscic acid		0.1 mg		
	Indole-3-Butyric Acid				0.1 mg
15	Sucrose		60 g	30 g	20 g
	Sorbitol	20 g			
	Bialaphos	1 mg			
	Gelrite	2 g			
	Phytagar		7 g	7 g	7 g
20	pH	5.8	5.8	5.8	5.8

<sup>1</sup> MS Salts – Sigma Plant culture Catalogue ref. M5519

Using this scheme, an average of 50% of the cultured embryos produced clones from which fertile transgenic plants are obtained. With this frequency of response, all 5000 fragments (with an average size of 150 kb) in the sorghum BIBAC library are readily introduced into the corn genome and each fragment can then be evaluated for its effect on the phenotype of elite corn inbreds and hybrids. Co-ordinate gene expression on the fragments in the transgenic plants allows sorghum traits to be evaluated in the transgenic corn. The requirement to evaluate specific sorghum sequences is met by the introduction of the entire sorghum genome in large fragments (averaging 150 kb in size) into the corn genome ensuring that all sorghum genes are tested.

EXAMPLE 4

Analysis for the Presence of Sorghum DNA Fragments in Corn

Analysis is carried out on a few transgenic plants to exemplify that indeed large intact fragments of sorghum DNA have been integrated into the corn genome via transformation with *Agrobacterium*.



In the transformation vector pSB12HBARGUS (see Examples 1 and 2) the marker genes *bar* and *gus* straddle the unique HindIII cloning site in which the sorghum DNA is cloned. Therefore, an indicator of the integration of the complete sorghum DNA fragment into the genome is the presence of both the *bar* and *gus* genes. Evaluation of the expression of these marker genes is easily done at the phenotypic level. *Bar* expression is demonstrated by the ability of the transgenic callus to grow on the selective agent bialaphos (see Example 3). In addition, a leaf paint assay is performed in which a 1% solution of the herbicide Liberty™ is painted on the leaf of a transgenic plant. The lack of herbicide damage indicates *bar* expression. Expression of *gus* is evaluated by a histochemical analysis. Callus or leaf tissue is incubated in the presence of the substrate X-gluc at the concentration of 0.5 mg/ml in 0.1 M sodium phosphate buffer pH 7.0 and 0.1% Triton-x-100 at 37° C for overnight. The presence of dark blue color in the tissue indicates *gus* expression.

The integration of sorghum DNA into the corn genome is detected by Southern blotting using conventional procedures. Briefly, total DNA is isolated from the transgenic plant, the DNA is cut with a restriction enzyme, the DNA is separated on a agarose gel using electrophoresis, and the DNA is transferred from the agarose gel onto a membrane that can then be probed with sorghum DNA used to transform the plant. In view of the high degree of homology between sorghum DNA and corn DNA, a unique region of sorghum DNA, such as 3' untranslated regions of genes, are used as probes for confirming the integration of sorghum DNA into the corn genome. Southern blotting confirms that sorghum DNA is integrated into corn DNA by this method.

## EXAMPLE 5

### Field Evaluation of Transgenic Corn Plants

Seed collected from transgenic regenerants which had been selfed or backcrossed to the parental line Stine inbred 963 is field planted and plants evaluated. In this example, transgenic events are evaluated using the segregation of the *bar* gene, one of the markers on the vector into which the sorghum DNA is inserted for transformation. If a marker gene is not utilized, transgenic events are evaluated using segregation of characteristics of interest. Those events which show Mendelian segregation for single copy insertion are chosen for further evaluation in the field. Segregants show variation for traits such as time to reach maturity, drought resistance, cold hardiness, increased pest

resistance, and stalk and root strength. Segregants with desirable changes are selected and selfed in a conventional breeding program. Plants homozygous for the transgenes are then crossed with tester inbreds and hybrid yield evaluated. All transgenic segregants potentially have an altered yield. In this manner, novel inbreds and hybrids are developed having improved agronomic traits resulting from the incorporation of sorghum DNA into the corn genome.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

#### LIST OF REFERENCES

- Allard, R.W. (1960). *Principles of plant breeding*, Wiley, New York.
- Chu, C.C. (1978). "The N6 medium and its application to anther culture of cereal crops." In *Proc. Symp. on Plant Tissue Culture*, Sci. Press, Beijing, pp 43 – 50.
- Ditta, G. et al. (1980). *Proc. Natl. Acad. Sci. USA* **77**:7347-7351.
- Fehr, W.R. (1987). *Principles of Cultivar Development*, Macmillan, New York
- Fromm, M.E. et al. (1986). *Nature* **312**:791-793.
- Green and Rhodes (1982). "Plant Regeneration in Tissue Culture of Maize." In *Maize for Biological Research*, W.F. Sheridan, ed., University of North Dakota, Grand Forks, North Dakota, pp 367-372.
- Hamilton, C.M. et al. (1996). "Stable transfer of intact high molecular weight DNA into plant chromosomes." *Proc. Natl. Acad. Sci. USA* **93**:997509979.
- Ishida, Y. et al. (1996). "High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*." *Nature Biotechnology* **14**:745-750.
- Klein, T.M. et al. (1987). *Nature* **327**:70-73.
- Lorz, H. et al. (1985)). *Mol. Gen. Genet.* **199**:178-182.
- Nester, E.W. et al. (1984). *Ann. Rev. Plant. Physiol.* **35**:387-413.
- Prioli and Söndahl (1989). "Plant Regeneration and Recovery of Fertile Plants from Protoplasts of Maize (*Zea Mays* L.)." *Bio/technology* **7**:589-594.
- Shillito et al. (1989). "Regeneration of Fertile Plants from Protoplasts of Elite Inbred Maize." *Bio/technology* **7**:581-587.

Simmonds, N.W. (1979). *Principles of crop improvement*, Longman, London, New York.

Sneep, J. and Hendrikson A.J.T.(1979). *Plant breeding perspectives*, Centre for Agricultural Publishing and Documentation, Wageningen.

5 Woo, S-S. et al. (1994). "Construction and characterization of a bacterial artificial chromosome library of *Sorghum bicolor*." *Nucl. Acids Res.* **22**:4922-4931.

Woo, S-S. et al. (1995). "Isolation of Megabase-Size DNA from *Sorghum* and Applications for Physical mapping and Bacterial and Yeast Artificial Chromosome Library Construction." *Plant Mol. Biol. Reporter* **13**:82-94.

U.S. Patent No. 4,665,030.

10 U.S. Patent No. 4,806,483.

U.S. Patent No. 4,843,005.

U.S. Patent No. 5,384,253.

U.S. Patent No. 5,489,520.

U.S. Patent No. 5,538,877.

15 U.S. Patent No. 5,550,318.

WHAT IS CLAIMED IS:

1. A method for obtaining a transgenic plant having an improved agronomic characteristic which comprises:
  - (a) preparing DNA fragments from DNA of a donor plant species;
  - (b) transforming plant cells of a recipient plant species with said DNA fragments;
  - (c) selecting transformed plant cells;
  - (d) regenerating plants from the transformed plant cells;
  - (e) harvesting seed from the regenerated plants;
  - (f) planting the harvested seed and growing the resultant plants;
  - (g) analyzing the plants for improved agronomic characteristics; and
  - (h) selecting plants having an improved agronomic characteristic.
2. The method of claim 1, wherein said DNA fragments are inserted into a vector prior to transforming plant cells.
3. The method of claim 2, wherein said DNA fragments inserted into a vector are inserted between two selectable markers.
4. The method of claim 1, wherein seed is harvested from regenerated plants which have been selfed.
5. The method of claim 1, wherein seed is harvested from regenerated plants which have been backcrossed to the recipient plant species.
6. The method of claim 1, wherein said plants having an improved agronomic characteristic are introduced into a breeding program to produce progeny of said plants, said progeny maintaining said improved agronomic characteristic.

7. The method of claim 1, wherein said donor plant is sorghum and said recipient plant is maize.
8. A transgenic plant produced by the process of claim 1.
9. A transgenic plant produced by the process of claim 2.
10. A transgenic plant produced by the process of claim 3.
- 5 11. A transgenic plant produced by the process of claim 4.
12. A transgenic plant produced by the process of claim 5.
13. A transgenic plant produced by the process of claim 6.
14. A transgenic plant produced by the process of claim 7.
- 10 15. A method for obtaining a transgenic plant having an improved agronomic characteristic which comprises:
  - (a) preparing DNA fragments from DNA of a donor plant species;
  - (b) inserting said DNA fragments into a vector;
  - (c) transforming plant cells of a recipient plant species with said vector containing said DNA fragments;
  - 15 (d) selecting transformed plant cells;
  - (e) regenerating plants from the transformed plant cells;
  - (f) harvesting seed from the regenerated plants;
  - (g) planting the harvested seed and growing the resultant plants;
  - (h) analyzing the plants for improved agronomic characteristics;
  - 20 (i) selecting plants having an improved agronomic characteristic;
  - (j) harvesting seed from said selected plants; and

(k) introducing seed from said selected plants into a breeding program to produce progeny of said plants, said progeny maintaining said improved agronomic characteristic.

16. The method of claim 15, wherein said DNA fragments inserted into a vector are inserted between two selectable markers.
17. The method of claim 15, wherein seed is harvested from regenerated plants which have been selfed.
18. The method of claim 15, wherein seed is harvested from regenerated plants which have been backcrossed to the recipient plant species.
19. A transgenic plant produced by the process of claim 15.
20. A transgenic plant produced by the process of claim 16.
21. A transgenic plant produced by the process of claim 17.
22. A transgenic plant produced by the process of claim 18.

TITLE OF THE INVENTION

TRANSGENIC PLANTS

ABSTRACT OF THE DISCLOSURE

5 The present invention is related to transgenic plants having improved traits and to a method for producing such plants. The method involves the incorporation of large fragments of DNA from a donor species of plant into a recipient species of plant and selecting plants with improved traits.

20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66  
67  
68  
69  
70  
71  
72  
73  
74  
75  
76  
77  
78  
79  
80  
81  
82  
83  
84  
85  
86  
87  
88  
89  
90  
91  
92  
93  
94  
95  
96  
97  
98  
99  
100  
101  
102  
103  
104  
105  
106  
107  
108  
109  
110  
111  
112  
113  
114  
115  
116  
117  
118  
119  
120  
121  
122  
123  
124  
125  
126  
127  
128  
129  
130  
131  
132  
133  
134  
135  
136  
137  
138  
139  
140  
141  
142  
143  
144  
145  
146  
147  
148  
149  
150  
151  
152  
153  
154  
155  
156  
157  
158  
159  
160  
161  
162  
163  
164  
165  
166  
167  
168  
169  
170  
171  
172  
173  
174  
175  
176  
177  
178  
179  
180  
181  
182  
183  
184  
185  
186  
187  
188  
189  
190  
191  
192  
193  
194  
195  
196  
197  
198  
199  
200  
201  
202  
203  
204  
205  
206  
207  
208  
209  
210  
211  
212  
213  
214  
215  
216  
217  
218  
219  
220  
221  
222  
223  
224  
225  
226  
227  
228  
229  
230  
231  
232  
233  
234  
235  
236  
237  
238  
239  
240  
241  
242  
243  
244  
245  
246  
247  
248  
249  
250  
251  
252  
253  
254  
255  
256  
257  
258  
259  
260  
261  
262  
263  
264  
265  
266  
267  
268  
269  
270  
271  
272  
273  
274  
275  
276  
277  
278  
279  
280  
281  
282  
283  
284  
285  
286  
287  
288  
289  
290  
291  
292  
293  
294  
295  
296  
297  
298  
299  
300  
301  
302  
303  
304  
305  
306  
307  
308  
309  
310  
311  
312  
313  
314  
315  
316  
317  
318  
319  
320  
321  
322  
323  
324  
325  
326  
327  
328  
329  
330  
331  
332  
333  
334  
335  
336  
337  
338  
339  
340  
341  
342  
343  
344  
345  
346  
347  
348  
349  
350  
351  
352  
353  
354  
355  
356  
357  
358  
359  
360  
361  
362  
363  
364  
365  
366  
367  
368  
369  
370  
371  
372  
373  
374  
375  
376  
377  
378  
379  
380  
381  
382  
383  
384  
385  
386  
387  
388  
389  
390  
391  
392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403  
404  
405  
406  
407  
408  
409  
410  
411  
412  
413  
414  
415  
416  
417  
418  
419  
420  
421  
422  
423  
424  
425  
426  
427  
428  
429  
430  
431  
432  
433  
434  
435  
436  
437  
438  
439  
440  
441  
442  
443  
444  
445  
446  
447  
448  
449  
450  
451  
452  
453  
454  
455  
456  
457  
458  
459  
460  
461  
462  
463  
464  
465  
466  
467  
468  
469  
470  
471  
472  
473  
474  
475  
476  
477  
478  
479  
480  
481  
482  
483  
484  
485  
486  
487  
488  
489  
490  
491  
492  
493  
494  
495  
496  
497  
498  
499  
500  
501  
502  
503  
504  
505  
506  
507  
508  
509  
510  
511  
512  
513  
514  
515  
516  
517  
518  
519  
520  
521  
522  
523  
524  
525  
526  
527  
528  
529  
530  
531  
532  
533  
534  
535  
536  
537  
538  
539  
540  
541  
542  
543  
544  
545  
546  
547  
548  
549  
550  
551  
552  
553  
554  
555  
556  
557  
558  
559  
560  
561  
562  
563  
564  
565  
566  
567  
568  
569  
570  
571  
572  
573  
574  
575  
576  
577  
578  
579  
580  
581  
582  
583  
584  
585  
586  
587  
588  
589  
590  
591  
592  
593  
594  
595  
596  
597  
598  
599  
600  
601  
602  
603  
604  
605  
606  
607  
608  
609  
610  
611  
612  
613  
614  
615  
616  
617  
618  
619  
620  
621  
622  
623  
624  
625  
626  
627  
628  
629  
630  
631  
632  
633  
634  
635  
636  
637  
638  
639  
640  
641  
642  
643  
644  
645  
646  
647  
648  
649  
650  
651  
652  
653  
654  
655  
656  
657  
658  
659  
660  
661  
662  
663  
664  
665  
666  
667  
668  
669  
670  
671  
672  
673  
674  
675  
676  
677  
678  
679  
680  
681  
682  
683  
684  
685  
686  
687  
688  
689  
690  
691  
692  
693  
694  
695  
696  
697  
698  
699  
700  
701  
702  
703  
704  
705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739  
740  
741  
742  
743  
744  
745  
746  
747  
748  
749  
750  
751  
752  
753  
754  
755  
756  
757  
758  
759  
760  
761  
762  
763  
764  
765  
766  
767  
768  
769  
770  
771  
772  
773  
774  
775  
776  
777  
778  
779  
780  
781  
782  
783  
784  
785  
786  
787  
788  
789  
790  
791  
792  
793  
794  
795  
796  
797  
798  
799  
800  
801  
802  
803  
804  
805  
806  
807  
808  
809  
810  
811  
812  
813  
814  
815  
816  
817  
818  
819  
820  
821  
822  
823  
824  
825  
826  
827  
828  
829  
830  
831  
832  
833  
834  
835  
836  
837  
838  
839  
840  
841  
842  
843  
844  
845  
846  
847  
848  
849  
850  
851  
852  
853  
854  
855  
856  
857  
858  
859  
860  
861  
862  
863  
864  
865  
866  
867  
868  
869  
870  
871  
872  
873  
874  
875  
876  
877  
878  
879  
880  
881  
882  
883  
884  
885  
886  
887  
888  
889  
890  
891  
892  
893  
894  
895  
896  
897  
898  
899  
900  
901  
902  
903  
904  
905  
906  
907  
908  
909  
910  
911  
912  
913  
914  
915  
916  
917  
918  
919  
920  
921  
922  
923  
924  
925  
926  
927  
928  
929  
930  
931  
932  
933  
934  
935  
936  
937  
938  
939  
940  
941  
942  
943  
944  
945  
946  
947  
948  
949  
950  
951  
952  
953  
954  
955  
956  
957  
958  
959  
960  
961  
962  
963  
964  
965  
966  
967  
968  
969  
970  
971  
972  
973  
974  
975  
976  
977  
978  
979  
980  
981  
982  
983  
984  
985  
986  
987  
988  
989  
990  
991  
992  
993  
994  
995  
996  
997  
998  
999  
1000  
1001  
1002  
1003  
1004  
1005  
1006  
1007  
1008  
1009  
1010  
1011  
1012  
1013  
1014  
1015  
1016  
1017  
1018  
1019  
1020  
1021  
1022  
1023  
1024  
1025  
1026  
1027  
1028  
1029  
1030  
1031  
1032  
1033  
1034  
1035  
1036  
1037  
1038  
1039  
1040  
1041  
1042  
1043  
1044  
1045  
1046  
1047  
1048  
1049  
1050  
1051  
1052  
1053  
1054  
1055  
1056  
1057  
1058  
1059  
1060  
1061  
1062  
1063  
1064  
1065  
1066  
1067  
1068  
1069  
1070  
1071  
1072  
1073  
1074  
1075  
1076  
1077  
1078  
1079  
1080  
1081  
1082  
1083  
1084  
1085  
1086  
1087  
1088  
1089  
1090  
1091  
1092  
1093  
1094  
1095  
1096  
1097  
1098  
1099  
1100  
1101  
1102  
1103  
1104  
1105  
1106  
1107  
1108  
1109  
1110  
1111  
1112  
1113  
1114  
1115  
1116  
1117  
1118  
1119  
1120  
1121  
1122  
1123  
1124  
1125  
1126  
1127  
1128  
1129  
1130  
1131  
1132  
1133  
1134  
1135  
1136  
1137  
1138  
1139  
1140  
1141  
1142  
1143  
1144  
1145  
1146  
1147  
1148  
1149  
1150  
1151  
1152  
1153  
1154  
1155  
1156  
1157  
1158  
1159  
1160  
1161  
1162  
1163  
1164  
1165  
1166  
1167  
1168  
1169  
1170  
1171  
1172  
1173  
1174  
1175  
1176  
1177  
1178  
1179  
1180  
1181  
1182  
1183  
1184  
1185  
1186  
1187  
1188  
1189  
1190  
1191  
1192  
1193  
1194  
1195  
1196  
1197  
1198  
1199  
1200  
1201  
1202  
1203  
1204  
1205  
1206  
1207  
1208  
1209  
1210  
1211  
1212  
1213  
1214  
1215  
1216  
1217  
1218  
1219  
1220  
1221  
1222  
1223  
1224  
1225  
1226  
1227  
1228  
1229  
1230  
1231  
1232  
1233  
1234  
1235  
1236  
1237  
1238  
1239  
1240  
1241  
1242  
1243  
1244  
1245  
1246  
1247  
1248  
1249  
1250  
1251  
1252  
1253  
1254  
1255  
1256  
1257  
1258  
1259  
1260  
1261  
1262  
1263  
1264  
1265  
1266  
1267  
1268  
1269  
1270  
1271  
1272  
1273  
1274  
1275  
1276  
1277  
1278  
1279  
1280  
1281  
1282  
1283  
1284  
1285  
1286  
1287  
1288  
1289  
1290  
1291  
1292  
1293  
1294  
1295  
1296  
1297  
1298  
1299  
1300  
1301  
1302  
1303  
1304  
1305  
1306  
1307  
1308  
1309  
1310  
1311  
1312  
1313  
1314  
1315  
1316  
1317  
1318  
1319  
1320  
1321  
1322  
1323  
1324  
1325  
1326  
1327  
1328  
1329  
1330  
1331  
1332  
1333  
1334  
1335  
1336  
1337  
1338  
1339  
1340  
1341  
1342  
1343  
1344  
1345  
1346  
1347  
1348  
1349  
1350  
1351  
1352  
1353  
1354  
1355  
1356  
1357  
1358  
1359  
1360  
1361  
1362  
1363  
1364  
1365  
1366  
1367  
1368  
1369  
1370  
1371  
1372  
1373  
1374  
1375  
1376  
1377  
1378  
1379  
1380  
1381  
1382  
1383  
1384  
1385  
1386  
1387  
1388  
1389  
1390  
1391  
1392  
1393  
1394  
1395  
1396  
1397  
1398  
1399  
1400  
1401  
1402  
1403  
1404  
1405  
1406  
1407  
1408  
1409  
1410  
1411  
1412  
1413  
1414  
1415  
1416  
1417  
1418  
1419  
1420  
1421  
1422  
1423  
1424  
1425  
1426  
1427  
1428  
1429  
1430  
1431  
1432  
1433  
1434  
1435  
1436  
1437  
1438  
1439  
1440  
1441  
1442  
1443  
1444  
1445  
1446  
1447  
1448  
1449  
1450  
1451  
1452  
1453  
1454  
1455  
1456  
1457  
1458  
1459  
1460  
1461  
1462  
1463  
1464  
1465  
1466  
1467  
1468  
1469  
1470  
1471  
1472  
1473  
1474  
1475  
1476  
1477  
1478  
1479  
1480  
1481  
1482  
1483  
1484  
1485  
1486  
1487  
1488  
1489  
1490  
1491  
1492  
1493  
1494  
1495  
1496  
1497  
1498  
1499  
1500  
1501  
1502  
1503  
1504  
1505  
1506  
1507  
1508  
1509  
1510  
1511  
1512  
1513  
1514  
1515  
1516  
1517  
1518  
1519  
1520  
1521  
1522  
1523  
1524  
1525  
1526  
1527  
1528  
1529  
1530  
1531  
1532  
1533  
1534  
1535  
1536  
1537  
1538  
1539  
1540  
1541  
1542  
1543  
1544  
1545  
1546  
1547  
1548  
1549  
1550  
1551  
1552  
1553  
1554  
1555  
1556  
1557  
1558  
1559  
1560  
1561  
1562  
1563  
1564  
1565  
1566  
1567  
1568  
1569  
1570  
1571  
1572  
1573  
1574  
1575  
1576  
1577  
1578  
1579  
1580  
1581  
1582  
1583  
1584  
1585  
1586  
1587  
1588  
1589  
1590  
1591  
1592  
1593  
1594  
1595  
1596  
1597  
1598  
1599  
1600  
1601  
1602  
1603  
1604  
1605  
1606  
1607  
1608  
1609  
1610  
1611  
1612  
1613  
1614  
1615  
1616  
1617  
1618  
1619  
1620  
1621  
1622  
1623  
1624  
1625  
1626  
1627  
1628  
1629  
1630  
1631  
1632  
1633  
1634  
1635  
1636  
1637  
1638  
1639  
1640  
1641  
1642  
1643  
1644  
1645  
1646  
1647  
1648  
1649  
1650  
1651  
1652  
1653  
1654  
1655  
1656  
1657  
1658  
1659  
1660  
1661  
1662  
1663  
1664  
1665  
1666  
1667  
1668  
1669  
1670  
1671  
1672  
1673  
1674  
1675  
1676  
1677  
1678  
1679  
1680  
1681  
1682  
1683  
1684  
1685  
1686  
1687  
1688  
1689  
1690  
1691  
1692  
1693  
1694  
1695  
1696  
1697  
1698  
1699  
1700  
1701  
1702  
1703  
1704  
1705  
1706  
1707  
1708  
1709  
1710  
1711  
1712  
1713  
1714  
1715  
1716  
1717  
1718  
1719  
1720  
1721  
1722  
1723  
1724  
1725  
1726  
1727  
1728  
1729  
1730  
1731  
1732  
1733  
1734  
1735  
1736  
1737  
1738  
1739  
1740  
1741  
1742  
1743  
1744  
1745  
1746  
1747  
1748  
1749  
1750  
1751  
1752  
1753  
1754  
1755  
1756  
1757  
1758  
1759  
1760  
1761  
1762  
1763  
1764  
1765  
1766  
1767  
1768  
1769  
1770  
1771  
1772  
1773  
1774  
1775  
1776  
1777  
1778  
1779  
1780  
1781  
1782  
1783  
1784  
1785  
1786  
1787  
1788  
1789  
1790  
1791  
1792  
1793  
1794  
1795  
1796  
1797  
1798  
1799  
1800  
1801  
1802  
1803  
1804  
1805  
1806  
1807  
1808  
1809  
1810  
1811  
1812  
1813  
1814  
1815  
1816  
1817  
1818  
1819  
1820  
1821  
1822  
1823  
1824  
1825  
1826  
1827  
1828  
1829  
1830  
1831  
1832  
1833  
1834  
1835  
1836  
1837  
1838  
1839  
1840  
1841  
1842  
1843  
1844  
1845  
1846  
1847  
1848  
1849  
1850  
1851  
1852  
1853  
1854  
1855  
1856  
1857  
1858  
1859  
1860  
1861  
1862  
1863  
1864  
1865  
1866  
1867  
1868  
1869  
1870  
1871  
1872  
1873  
1874  
1875  
1876  
1877  
1878  
1879  
1880  
1881  
1882  
1883  
1884  
1885  
1886  
1887  
1888  
1889  
1890  
1891  
1892  
1893  
1894  
1895  
1896  
1897  
1898  
1899  
1900  
1901  
1902  
1903  
1904  
1905  
1906  
1907  
1908  
1909  
1910  
1911  
1912  
1913  
1914  
1915  
1916  
1917  
1918  
1919  
1920  
1921  
1922  
1923  
1924  
1925  
1926  
1927  
1928  
1929  
1930  
1931  
1932  
1933  
1934  
1935  
1936  
1937  
1938  
1939  
1940  
1941  
1942  
1943  
1944  
1945  
1946  
1947  
1948  
1949  
1950  
1951  
1952  
1953  
1954  
1955  
1956  
1957  
1958  
1959  
1960  
1961  
1962  
1963  
1964  
1965  
1966  
1967  
1968  
1969  
1970  
1971  
1972  
1973  
1974  
1975  
1976  
1977  
1978  
1979  
1980  
1981  
1982  
1983  
1984  
1985  
1986  
1987  
1988  
1989  
1990  
1991  
1992  
1993  
1994  
1995  
1996  
1997  
1998  
1999  
2000  
2001  
2002  
2003  
2004  
2005  
2006  
2007  
2008  
2009  
2010  
2011  
2012  
2013  
2014  
2015  
2016  
2017  
2018  
2019  
2020  
2021  
2022  
2023  
2024  
2025  
2026  
2027  
2028  
2029  
2030  
2031  
2032  
2033  
2034  
2035  
2036  
2037  
2038  
2039  
2040  
2041  
2042  
2043  
2044  
2045  
2046  
2047  
2048  
2049  
2050  
2051  
2052  
2053  
2054  
2055  
2056  
2057  
2058  
2059  
2060  
2061  
2062  
2063  
2064  
2065  
2066  
2067  
2068  
2069  
2070  
2071  
2072  
2073  
2074  
2075  
2076  
2077  
2078  
2079  
2080  
2081  
2082  
2083  
2084  
2085  
2086  
2087  
2088  
2089  
2090  
2091  
2092  
2093  
2094  
2095  
2096  
2097  
2098  
2099  
2100  
2101  
2102  
2103  
2104  
2105  
2106  
2107  
2108  
2109  
2110  
2111  
2112  
2113  
2114  
2115  
2116  
2117  
2118  
2119  
2120  
2121  
2122  
2123  
2124  
2125  
2126  
2127  
2128  
2129  
2130  
2131  
2132  
2133  
2134  
2135  
2136  
2137  
2138  
2139  
2140  
2141  
2142  
2143  
2144  
2145  
2146  
2147  
2148  
2149  
2150  
2151  
2152  
2153  
2154  
2155  
2156  
2157  
2158  
2159  
2160  
2161  
2162  
2163  
2164  
2165  
2166  
2167  
2168  
2169  
2170  
2171  
2172  
2173  
2174  
2175  
2176  
2177  
2178  
2179  
2180  
2181  
2182  
2183  
2184  
2185  
2186  
2187  
2188  
2189  
2190  
2191  
2192  
2193  
2194  
2195  
2196  
2197  
2198  
2199  
2200  
2201  
2202  
2203  
2204  
2205  
2206  
2207  
2208  
2209  
2210  
2211  
2212  
2213  
2214  
22

## DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought, on the invention entitled TRANSGENIC PLANTS, the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims.

I acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

No claim for foreign priority benefits under Title 35, United States Code, § 119 is made and no corresponding foreign applications for patent or inventor's certificate having a filing date before that of the present application have been filed.

I hereby appoint the following attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to ROTHWELL, FIGG, ERNST & KURZ, p.c., Suite 701-E, 555 13th Street, N.W., Washington, D.C 20004

G. Franklin Rothwell, Reg. No. 18,125  
Barbara G. Ernst, Reg. No. 30,377  
Bart G. Newland, Reg. No. 31,282  
Celine Jimenez Crowson, Reg. No. 40,357  
Robert J. Jondle, Reg. No. 33,915  
Michael G. Sullivan, Reg. No. 35,377  
Don M. Kerr, Reg. No. 22,720  
Kenneth M. Fagin, Reg. No. 37,615  
Jeffrey W. Rennecker, Reg. No. 40,784  
Lisa Fahien Uldrich, Reg. No. 38,875

E. Anthony Figg, Reg. No. 27,195  
George R. Repper, Reg. No. 31,414  
Vincent M. DeLuca, Reg. No. 32,408  
Joseph A. Hynds, Reg. No. 34,627  
Jeffrey I. Ihnen, Reg. No. 28,957  
Mark I. Bowditch, Reg. No. 40,315  
Stephen B. Parker, Reg. No. 36,631  
Minaksi Bhatt, Reg. No. 35,447  
Stephen A. Saxe, Reg. No. 38,609

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1. **Inventor's Signature** Herbert Martin Wilson **Date** 8/20/98  
Inventor's Name Herbert Martin Wilson Citizenship United States  
Residence (City) Ames, Story County, Iowa  
Mailing Address 1915 Stevenson Drive, Ames, IA 50010

2. **Inventor's Signature** Bruce Marvin Held **Date** 8-20-98  
Inventor's Name Bruce Marvin Held Citizenship United States  
Residence (City) Ames, Story County, Iowa  
Mailing Address 5449 Frost Drive, Ames, IA 50010

3. **Inventor's Signature** Harry Henry Stine **Date** 19 Nov 1998  
Inventor's Name Harry Henry Stine Citizenship United States  
Residence (City) Adel, Dallas County, Iowa  
Mailing Address 2225 Laredo Trail, Adel, IA 50003



Applicant or Patentee: HERBERT MARTIN WILSON, et al.  
Serial or Patent No.: 09/  
Filed or Issued:  
For: "TRANSGENIC PLANTS"

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 CFR 1.9(f) and 1.27(C)) - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☒ the owner of the small business concern identified below:  
☐ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Stine Biotechnology  
ADDRESS OF CONCERN: 2501 N. Loop Drive, Suite 611, Ames, Iowa 50010

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled "TRANSGENIC PLANTS" by inventors Herbert Martin Wilson, Bruce Marvin Held and Harry Stine, described in

- ☒ the specification filed herewith  
☐ application serial no. 09/\_\_\_\_\_, filed \_\_\_\_\_  
☐ patent no. \_\_\_\_\_, issued \_\_\_\_\_

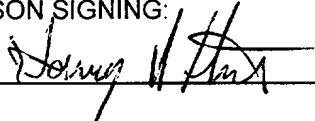
If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).  
\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME \_\_\_\_\_ ADDRESS \_\_\_\_\_  
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any application issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: HARRY STINE  
TITLE OF PERSON OTHER THAN OWNER: President  
ADDRESS OF PERSON SIGNING: 2501 N. Loop Drive, Ames, Iowa 5000

SIGNATURE: 

Date 19 Nov 1998